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Abstract: Osteosarcoma is a highly aggressive bone cancer and the second most frequent cause of cancer-associated death in childhood and adolescence. Pulmonary metastases account for the high mortality rate in osteosarcoma patients. Therefore, novel therapeutic approaches, efficiently restraining the metastatic disease, are mandatory for a significant improvement of the currently poor patients' survival. Although initial studies with antibodies targeting insulin-like growth factor receptor (IGF-IR) showed promising potential for the treatment of patients with bone and soft tissue sarcomas, phase II clinical trials revealed variable results, which implied activation of alternative signaling pathways leading to therapy resistance. Since a cross-talk between IGF-IR and the epidermal growth factor receptor (EGFR) has been demonstrated in several cancer types, co-targeting of these two receptors was considered in the present study as a valuable therapeutic strategy to overcome single-agent treatment resistance in osteosarcoma. The effects of IGF-IR and/or EGFR targeting by intraperitoneal administration of the monospecific IGF-IR antibody R1507 or the EGFR antibody Cetuximab or the bispecific IGF-IR/EGFR antibody XGFR* on primary tumor growth and pulmonary metastasis were investigated in an intratibial human xenograft osteosarcoma mouse model. In vitro functional assays demonstrated that targeting IGF-IR and EGFR didn't affect osteosarcoma cell viability, but inhibited ligand-activated intracellular signaling and cell migratory capacity. The blocking potential of ligand-induced signaling in vitro was similar for all antibodies, but, in vivo, only XGFR* treatment significantly inhibited intratibial primary tumor growth and pulmonary metastasis. The therapeutic response to XGFR* was associated with an infiltration of innate immune system effector cells into the tumor microenvironment. Taken together, our study highlights the bispecific anti-IGF-IR/EGFR antibody XGFR* as an innovative promising effective candidate for the treatment of metastatic osteosarcoma and provides the rationale for future clinical studies.

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Original Article

A bispecific antibody targeting IGF-IR and EGFR has tumor and metastasis suppressive activity in an orthotopic xenograft osteosarcoma mouse model

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Keywords: Osteosarcoma, metastasis, targeted therapy, xenograft mouse model, bispecific antibody, XGFR*, R1507, Cetuximab

Introduction

Osteosarcoma is the most common primary malignant bone cancer in children and young adults [1]. With predominant metastasis to the lungs, osteosarcoma is a highly aggressive disease, reflected by the fact that 20% of patients have detectable metastases at diagnosis and 80% of the patients initially presenting with localized disease subsequently develop metastases [2]. Multiple efforts to improve the treatment efficacy in patients with metastastic disease unfortunately failed to improve the survival rates, which plateaued at 20% over the past decades. Hence, it is of outmost importance to develop novel innovative therapeutic strategies that predominantly and more effectively suppress metastasis, the major cause of death of osteosarcoma patients.

Potential targets include the insulin-like growth factor-1 receptor (IGF-IR) and the epidermal growth factor receptor (EGFR). They are cell surface-located transmembrane tyrosine kinases that are frequently overexpressed in a variety of human cancers [3, 4]. Upon activation, IGF-IR and EGFR affect tumor growth and progression by regulation of tumor cell proliferation, inhibition of apoptosis and activation of angiogenesis via the PI3K-Akt and RAS-RAF-MAPK pathways.

IGF-IR has been implicated in the pathogenesis of sarcomas. It has been shown that human osteosarcoma cells are mitogenically respon-

sive to IGF-1 *in vitro*. IGF-1 receptor stimulation also contributed to increased invasive activity of human and canine osteosarcoma cells [5]. Moreover, hypophysectomy in mice, lowering IGF-1 levels, reduced primary tumor growth and metastasis of murine osteosarcoma [6, 7]. Targeting various sarcoma types with different IGF-IR antibodies has already been performed [8]. Most of the preclinical studies, however, focused only on the effects on primary tumor growth in subcutaneous tumor models in mice, but, importantly, effects on metastatic dissemination have so far not been investigated in sarcomas in general and in osteosarcoma in particular. [9-11]. Hence, in the present study, we focused on the effects of IGF-IR targeting on pulmonary metastasis of human osteosarcoma cell line-derived intratibial tumors in mice. We intraperitoneally treated mice with R1507 antibody, a fully human IgG1-type monoclonal antibody directed against the human IGF-IR [11]. Phase I and phase II clinical trials in patients with solid tumors showed that R1507 is well tolerated and shows tumor suppressive activity especially in Ewing's sarcoma [12, 13]. R1507 had limited activity in patients with bone and soft tissue sarcomas and considerable variability in the outcome of patients in response to anti-IGF-IR therapy was observed [14]. Low response rates, already described in a variety of cancer types, were presumably due to acquired resistance mechanisms such as activation of alternative signaling pathways. Corresponding signaling molecules may include MET, platelet-derived growth factor (PDGFR) or EGFR [15-17].

The biological relevance of EGFR for osteosarcoma pathogenesis is controversially discussed [18, 19]. Interestingly, targeting human EGFR with Cetuximab, a chimeric mouse/human monoclonal antibody of the IgG1 subclass, together with natural killer cells has been employed to kill human osteosarcoma EGFR-expressing cells even at low receptor density [20]. A phase II clinical trial of Cetuximab in patients with metastatic or locally advanced soft tissue or bone sarcoma reported that this antibody has a good safety profile but is not effective as a single agent in advanced sarcoma [21]. It is, however, important to note that this study was not conclusive since only two osteosarcoma patients with tumor tissue lacking EGFR expression were included. Therefore, further studies with Cetuximab in osteosarcoma patients are needed. Interestingly, Cetuximab has so far not been investigated in osteosarcoma animal models.

A cross-talk between IGF-IR and EGFR has been demonstrated in several cancer types [22, 23]. In addition, EGFR has been shown to be involved in the regulation of the secretion of CCN1 [24], a protein that was recently found to have metastasis promoting activity in osteosarcoma [25]. Thus, combined targeting of IGF-IR and EGFR in osteosarcoma needs to be investigated. Bispecific antibodies that simultaneously target two different antigens are nowadays considered as a next generation of effective molecules for cancer therapy [26]. In addition to simultaneous targeting of two different cell surface receptors on tumor cells and thereby blocking two different signaling pathways, bispecific antibodies have also the valuable therapeutic property to redirect specific immune effector cells to tumor cells, which leads to enhanced tumor cell killing [27].

In order to simultaneously block both IGF-IR and EGFR, we made use of a novel bispecific antibody XGFR*, that was found to have a potent tumor suppressive activity in an orthotopic xenograft mouse model of pancreatic cancer [28]. This antibody is an affinity-matured antibody targeting IGF-IR and EGFR based on the glycoengineered bispecific IgG antibody XGFR, previously described to have *in vitro* and *in vivo* anti-tumor activity in several xenograft mouse tumor models including pancreatic, lung and colorectal cancer models [29]. XGFR*, a highly functionally improved molecule with maximal monovalent binding of IGF-IR and EGFR, bears afucosylated Fc-portion optimal to provoke antibody-dependent cell-mediated cytotoxicity (ADCC).

The main aim of the here presented study was to investigate and compare in an intratibial human xenograft osteosarcoma mouse model the primary tumor and metastasis suppressive efficacy of monospecific IGF-IR- or EGFR-blocking antibodies administered alone or in combination and of a bispecific IGF-R/EGFR antibody.

Materials and methods

Cell culture and antibodies

The human osteosarcoma cell lines SaOS-2 (HTB-85), HOS (CRL-1543) and 143-B (CRL-

Table 1. Primer pairs used in qRT-PCR

Gene Name (human)	Forward and Reverse Primer Sequence
IGF-1	Fwd: AGCCCCCATCTACCAACAAG
	Rev: GGTGGCATGTCACTCTTCACT
TGF-α	Fwd: CCCTGTTCGCTCTGGGTATT
	Rev: GTGGGAATCTGGGCAGTCAT
EGF	Fwd: ATGTGTGCAGAGGGATACGC
	Rev: CTACAGGGCACGTGCAGTAA
GAPDH	Fwd: AAGGCTGGGGCTCATTTGCAGG
	Rev: AGTTGGTGGTGCAGGAGGCA

8303) cells were obtained from American Type Culture Collection (ATCC) (Rockville, MD). LM5 cells were kindly provided by E.S. Kleinerman (M.D. Anderson Cancer Center, Houston, TX), HUO9 and HUO9-M132 (M132) cells by M. Tani (National Cancer Center Hospital, Tokyo, Japan), MG63 cells by G. Sarkar (Mayo Clinic, Rochester, MN) and MG63-M8 (M8) cells by W.T. Zhu (Tongji Hospital, Huazhong University of Science and Technology, Wuhan, China). 143-B and SaOS-2 cells were stably transduced with a LacZ gene revealing SaOS-2/LacZ and 143-B/LacZ cells. They were selected as previously reported [30-32] and cultured in tissue culture medium containing DMEM (4.5 g/l glucose)/HamF12 (1:1) medium (Invitrogen, Carlsbad, CA) supplemented with 10% heat inactivated fetal bovine serum (FBS; Lubio Science, Lucerne; Switzerland), referred to as complete medium in the text. The cells were kept at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The cell line authentication was done by short tandem repeat DNA profiling (Microsynth; Balgach, Switzerland) with a PowerPlex®16HS system (Promega. Madison, WI) and by comparison with the German Collection of Microorganisms and Cell Cultures Database (DSMZ). SaOS-2/LacZ and 143-B/LacZ cells were used in *in vitro* functional assays. In order to enable visualization of tumor cells within mouse tissues *in vivo* and *ex vivo*, 143-B/LacZ cells were additionally transduced with an mCherry gene (143-B/mCherry/LacZ cells) as described recently [33]. Roche Diagnostics GmbH (pRED Discovery Oncology, RICM, Germany) kindly provided R1507 (a fully humanized IgG1 monoclonal antibody directed against the extracellular portion of IGF-1R at a stock concentration of 25.6 mg/ml), XGFR* (RO6842831, a bispecific anti-IGF-1R/EGFR antibody at a stock concentration of 5.9 mg/ml) and Cetuximab (Erbitux; Merck KGaA, Darmstadt, Germany), a chimeric IgG1 type monoclonal anti-EGFR antibody at a stock concentration of 5 mg/ml.

RNA extraction, cDNA synthesis and quantitative real-time polymerase chain reaction (qPCR)

Total RNA was isolated with an RNeasy Mini Kit (Qiagen, Hilden, Germany; 74104) as described in the protocol provided by the manufacturer. RNA concentrations and purity were analyzed by OD 260/280 measurements in a NanoDrop device (Thermo Scientific, Waltham, MA). 1 µg of total RNA was transcribed to cDNA with a High-Capacity cDNA Reverse Transcription Kit with Rnase Inhibitor (Applied Biosystems, Foster City, CA; 4374966) following the manufacturer's instructions. Real-time qPCR was conducted on cDNA equivalent to 10 ng of starting RNA using a Power SYBR Green PCR Master Mix (Applied Biosystems, 4367659) with a StepOne-Plus Real-Time PCR System (Applied Biosystems). Primers used are listed in **Table 1**. The results obtained from three independent RNA preparations of individual cell lines were analyzed with StepOne Software version 2.1 (Applied Biosystems). Relative expression levels of individual transcripts were calculated by the comparative (ΔΔCT) method and normalized to GAPDH.

Stimulation and downregulation experiments and Western blotting

In stimulation experiments, cells were cultured for 36 hours in medium depleted of FBS. After starvation the cells were left untreated or preincubated for 1 hour with control IgG (Jackson Immunoresearch Labs Inc, West Grove, PA; 009-000-003; 20 µg/ml) or with R1507 (2 or 20 µg/ml) or Cetuximab (2 or 20 µg/ml) or with a combination of R1507 and Cetuximab (20 µg/ml) or with XGFR* (2 or 20 µg/ml) prior to stimulation with IGF-1 (10 ng/ml) or EGF (10 ng/ml) or the combination of both growth factors.

In order to investigate effects of IGF-IR- and EGFR-targeting antibodies on IGF-IR and EGFR protein expression, the cells were incubated in complete tissue culture medium alone for 24 hours or in medium containing 1 µg/ml of

R1507 or Cetuximab or XFGR* for 1, 2, 6 or 24 hours.

The preparation of protein cell extracts and Western blot analysis were conducted as previously described [32]. Antibodies used were anti-IGF-IR (3027, dilution 1:1000), anti-EGFR (4267, dilution 1:1000), anti-phospho-Erk (9101, dilution 1:1000), anti-total-Erk (9102, dilution 1:1000), anti-phospho-Akt (4060, dilution 1:1000), anti-total-Akt (9272, dilution 1:1000) purchased from Cell Signaling Technology (Beverley, MA), anti-GAPDH (FL-335, dilution 1:3000) and HRP-conjugated secondary antibodies (sc2054, dilution 1:5000) were obtained from Santa Cruz Biotechnologies (Dallas, TX) and antibodies to β-actin (dilution 1:10000) from Merck Millipore (Darmstadt, Germany).

Immunocytochemistry

Confluent 143-B cells seeded on round cover slips were fixed with 2% formalin for 10 minutes at room temperature (RT), quenched with complete cell culture medium for 1 hour at RT followed by incubation with IgG control, R1507, Cetuximab or XGFR* (2 μg/ml) for 2 hour at RT in PBS, 0.1% BSA. After three washes, the cells were incubated with Alexa546 anti-human IgG (Invitrogen; 1:400) for 30 minutes at RT and after three washes slides were mounted with ImmuMount (Thermo Shandon; Pittsburgh, PA). Cells were viewed with a Zeiss Axiovert.Z1 fluorescence microscope equipped with a DsRed fluorescence filter set, a 10 × objective and an AxioCam MRm camera (Carl Zeiss AG, Feldbach, Switzerland).

Cell viability assay

3×10^3 SaOS-2 or $0.5\text{-}1 \times 10^3$ 143-B cells per well were seeded in tissue culture medium in 96-well plates and allowed to adhere overnight. The cells were then incubated in triplicates with increasing concentrations of R1507, Cetuximab or XGFR* antibodies for 72 hours. Following antibody treatment, the cells were incubated with 10 μl/well of WST-1 reagent (Roche, Rotkreuz, Switzerland) for 3 hours and the cell viability was then assessed as previously described [34]. Three independent experiments were performed.

Transwell migration assay

The migratory properties of osteosarcoma cells were assessed in a transwell migration assay as reported previously [32]. Briefly, 10^4 143-B or 2×10^4 SaOS-2 cells were seeded in the absence or presence of R1507 (1 μg/ml) or Cetuximab (1 μg/ml) or the combination of R1507 and Cetuximab (1 μg/ml) or of XGFR (1 μg/ml) antibodies diluted in serum-free medium and allowed to migrate for 6 hours or 24 hours towards FBS-containing medium, respectively. The number of migrated cells in the analyzed area was determined with ImageJ software (http://rsb.info.nih.gov/ij/) and the total number of migrated cells per well was then calculated. The number of migrated cells treated with targeting antibodies was normalized to the number of migrated untreated cells set to 100%. The experiments were done in duplicates and repeated three times.

In vivo treatment study

The Ethics Committee of the Canton Zurich Veterinary Office approved the animal study that was subsequently conducted in accordance with the Swiss Animal Protection Law. Eight to ten weeks old female SCID/CB17 immunocompromised mice, purchased from Charles River Laboratories, were intratibially injected with 10^5 143-B/mCherry/LacZ cells in 10 μl of PBS/0.05% EDTA on day 0. Primary tumor development was examined weekly by X-ray and caliper measurements as previously described [35]. Ten days after tumor cell injection (TCI), when the primary tumor became detectable by X-Ray in all animals, the mice were randomly distributed according to their weight into 5 groups and the intraperitoneal weekly treatment with antibodies was initiated. The control group received the vehicle (physiological saline, n=12), whereas the treatment groups were injected with R1507 (10 mg/kg of body weight, n=10), or Cetuximab (10 mg/kg, n=12), or a combination of R1507 and Cetuximab (10 mg/kg + 10 mg/kg, n=12) or with XGFR* (20 mg/kg, n=10). The XGFR* antibody concentration of 20 mg/kg was used in order to ensure equimolar concentrations of dosing between XGFR* and the combination of R1507 and Cetuximab. The animals were sacrificed 27 days after TCI and the primary tumors and *in situ* perfused lungs were dissected, X-Gal stained and the numbers of metastases on the lung surface were quantified as reported [31, 35].

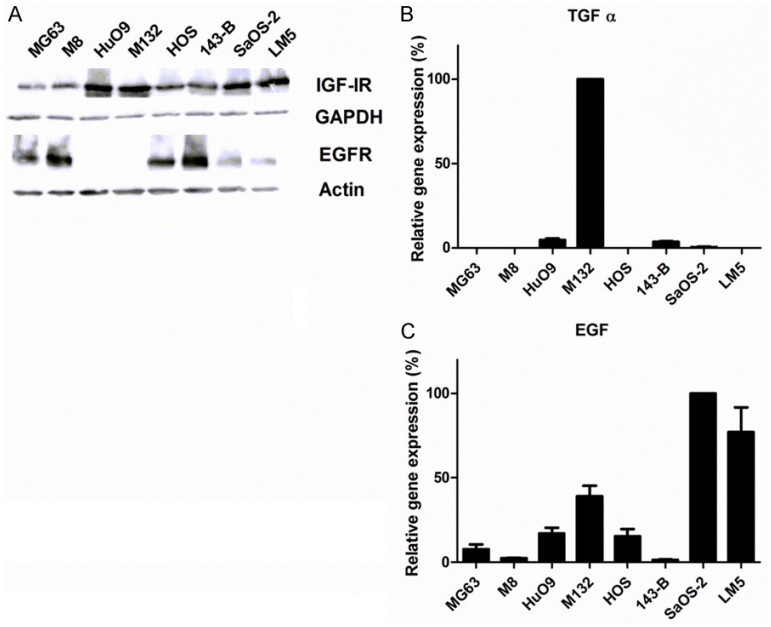

Figure 1. Expression of IGF-IR and EGFR and of EGFR ligands in human osteosarcoma cell lines. (A) IGF-IR and EGFR protein levels in whole cell extracts were examined by Western blot analysis using antibodies to IGF-IR or EGFR and to GAPDH or Actin as protein loading controls. Representative Western blots of three independent experiments. The levels of mRNA encoding TGFα (B) and EGF (C) were analyzed by quantitative RT-PCR and normalized to those of GAPDH in total RNA extracts. The relative expression levels were plotted as % of the maximal value. Results are presented as the mean ± SEM of at least 3 independent experiments.

Immunohistochemistry

Pieces of dissected primary tumors were immediately embedded in Tissue-Tek® O.C.T. Compound (Sakura Finetek, Torrance, CA), frozen on dry ice and kept at -80°C prior to cutting. The presence of natural killer (NK) cells was investigated by immunohistochemistry on frozen tissue sections according to standard protocols using a pan-NK anti-mouse CD49b antibody (BD Pharmingen, Allschwil, Switzerland; dilution 1:40). Slides incubated with secondary antibody alone served as negative controls. Cell nuclei were counterstained with hematoxylin. Primary tumor sections from three mice per group were analyzed. At least 3 images of randomly selected areas per tumor section were taken with an AxioCam MRc camera connected to the Zeiss Observer.Z1 inverted microscope (Carl Zeiss AG, Feldbach, Switzerland) set at 10 × magnification. Positive NK staining (red) and negative (purple) staining were separated using Fiji software [36]. The area percentage of the stain was defined as positive stained area (number of red pixels) over total tissue area (number of red and purple pixels).

Statistical analysis

Statistical significance of differences between the experimental groups was determined using a one or two-way ANOVA test with Dunnett's Multiple Comparison or Newman-Keuls Multiple Comparison post-tests and $P<0.05$ was considered significant. All analyses were done with GraphPad Prism Version 5.01 (GraphPad Software, Inc., La Jolla, CA). The results are presented as means ± standard error of the mean (SEM).

Results

Expression analysis of IGF-IR and EGFR and corresponding ligands in established human osteosarcoma cell lines

With the purpose to select the appropriate cell lines for the studies investigating in an intratibial osteosarcoma mouse model primary tumor and metastasis suppressive effects of IGF-IR- and EGFR-targeting antibodies including the bispecific XFGR*, we first studied the expression of IGF-IR and EGFR in a panel of eight human osteosarcoma cell lines. Western blot analysis of whole cell extracts revealed wide expression of IGF-IR in all osteosarcoma cell lines investigated (**Figure 1A**, upper panel). EGFR, on the other hand, was only expressed in six out eight cell lines (**Figure 1A**, lower panel). Interestingly, the highest expression of IGF-IR was observed in cell lines in which the expression of EGFR was non-detectable or low (HUO9, M132, SaOS-2, LM5). In order to identify the cell lines with possible autocrine IGF-IR and EGFR signaling axes, we investigated the expression of respective ligands at the mRNA level by qPCR. The levels of IGF-1 mRNA were very low or undetectable in all cell lines analyzed (40 cycles; data not shown). The level of expression of mRNA encoding TGFα, a ligand of EGFR, was found highest in the M132 cell line (**Figure 1B**). Overall the levels of EGF mRNA expression in the cell lines

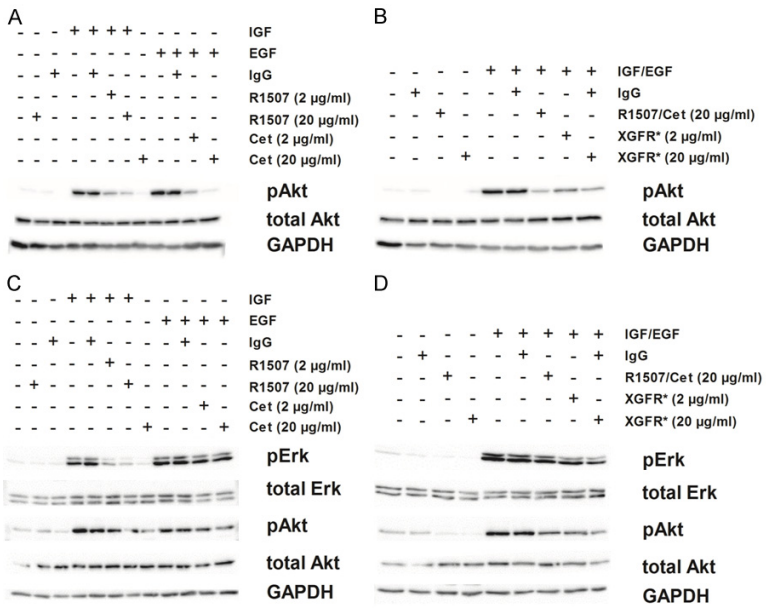

Figure 2. Effects of R1507/Cetuximab/XGFR* on IGF-1/EGF-induced signaling in 143-B and SaOS-2 cells. Serum-starved (36 hours) 143-B (A, B) or SaOS-2 (C, D) cells were left untreated or were pre-incubated for 1 hour with control IgG (20 µg/ml) or with indicated concentrations of R1507 or Cetuximab (A, C) or a combination of R1507 and Cetuximab or with XGFR* (B, D) prior to stimulation with IGF-1 (10 ng/ml) or EGF (10 ng/ml) or the combination of both growth factors. Cell lysates were analyzed by Western blots, using indicated antibodies. Representative Western blots of three independent experiments.

investigated were low, as indicated by Ct values for EGF transcripts that varied between 30 and 35 cycles. Among the eight cell lines the SaOS-2 and LM5 cell lines had the highest EGF transcript levels (**Figure 1C**).

In summary, IGF-IR and EGFR were found to be expressed in all or in the vast majority, respectively, of the here investigated human osteosarcoma cell lines. Non-detectable expression of IGF-1 encoding mRNA and the overall low expression of TGFα and EGF mRNA transcripts in most of the investigated cell lines implicate that autocrine signaling loops are largely inexistent in the majority of the osteosarcoma cell lines analyzed in the present study.

Effects of antibody-based targeting of IGF-IR and EGFR on their downstream signaling in vitro

One of the mechanisms of antibody-based therapy of cancer involves antibody-mediated blocking of ligand/receptor interaction on tumor cells and, as a consequence, inhibition or complete abrogation of the receptor signaling [37]. Therefore, we next evaluated the ability of R1507, of Cetuximab, of the combination of R1507 and Cetuximab or of XGFR* to inhibit ligand-activated IGF-IR and EGFR-mediated signaling. The 143-B and SaOS-2 cell lines were chosen for the here described *in vitro* experiments because they co-express IGF-IR and EGFR. Pre-incubation of serum-starved 143-B cells with R1507, Cetuximab or XGFR* inhibited in a dose-dependent manner IGF-1- and EGF-stimulated signaling, indicated by remarkable inhibition of the phosphorylation of Akt, one of the major effector components in the IGF-IR and EGFR signaling cascades (**Figure 2A**, **2B**). R1507 and Cetuximab together inhibited Akt phosphorylation accordingly and to a similar extent as the bispecific antibody XGFR* (**Figure 2B**). Given the fact that 143-B cells were generated through Ki-Ras transformation [38] and, therefore, exhibit constitutive Erk signaling, we did not investigate Erk phosphorylation upon IGF-IR and EGFR blocking. In serum-starved SaOS-2 cells, however, pre-incubation with R1507 provoked a prominent inhibition of IGF-1-stimulated Erk phosphorylation, but minimally affected Akt phosphorylation (**Figure 2C**). Conversely, Cetuximab did not affect EGF-stimulated Erk signaling, but, when used at a high concentration, inhibited Akt phosphorylation (**Figure 2C**). The combination of R1507 and Cetuximab had minimal effects on IGF-1- and EGF-stimulated signaling in SaOS-2 cells (**Figure 2D**). In contrast, as shown in **Figure 2D**, pre-incubation of SaOS-2 cells with XGFR* led to lower levels of both phosphorylated Erk and Akt compared to control upon stimulation with IGF-1 and EGF. Importantly, incubation of the cells with the individual antibodies and IgG alone (in the absence of ligands) did not stimulate any signaling in the two cell lines investigated. Moreover and as expected, in the absence and presence of IgG (control), IGF-1 or EGF-provoked signaling was indistinguishable in both cell lines.

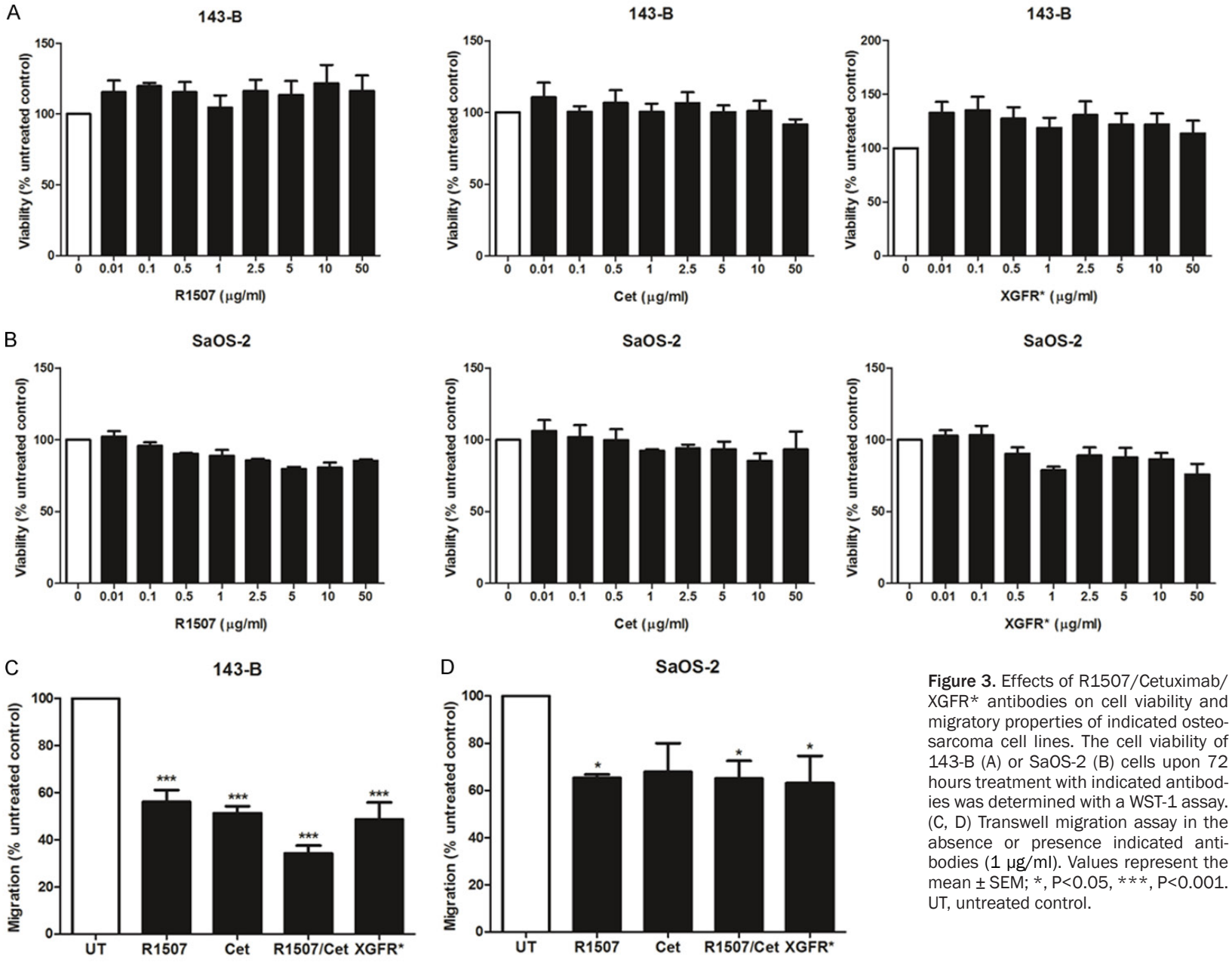

Figure 3. Effects of R1507/Cetuximab/XGFR* antibodies on cell viability and migratory properties of indicated osteosarcoma cell lines. The cell viability of 143-B (A) or SaOS-2 (B) cells upon 72 hours treatment with indicated antibodies was determined with a WST-1 assay. (C, D) Transwell migration assay in the absence or presence indicated antibodies (1 μg/ml). Values represent the mean ± SEM; *, P<0.05, ***, P<0.001. UT, untreated control.

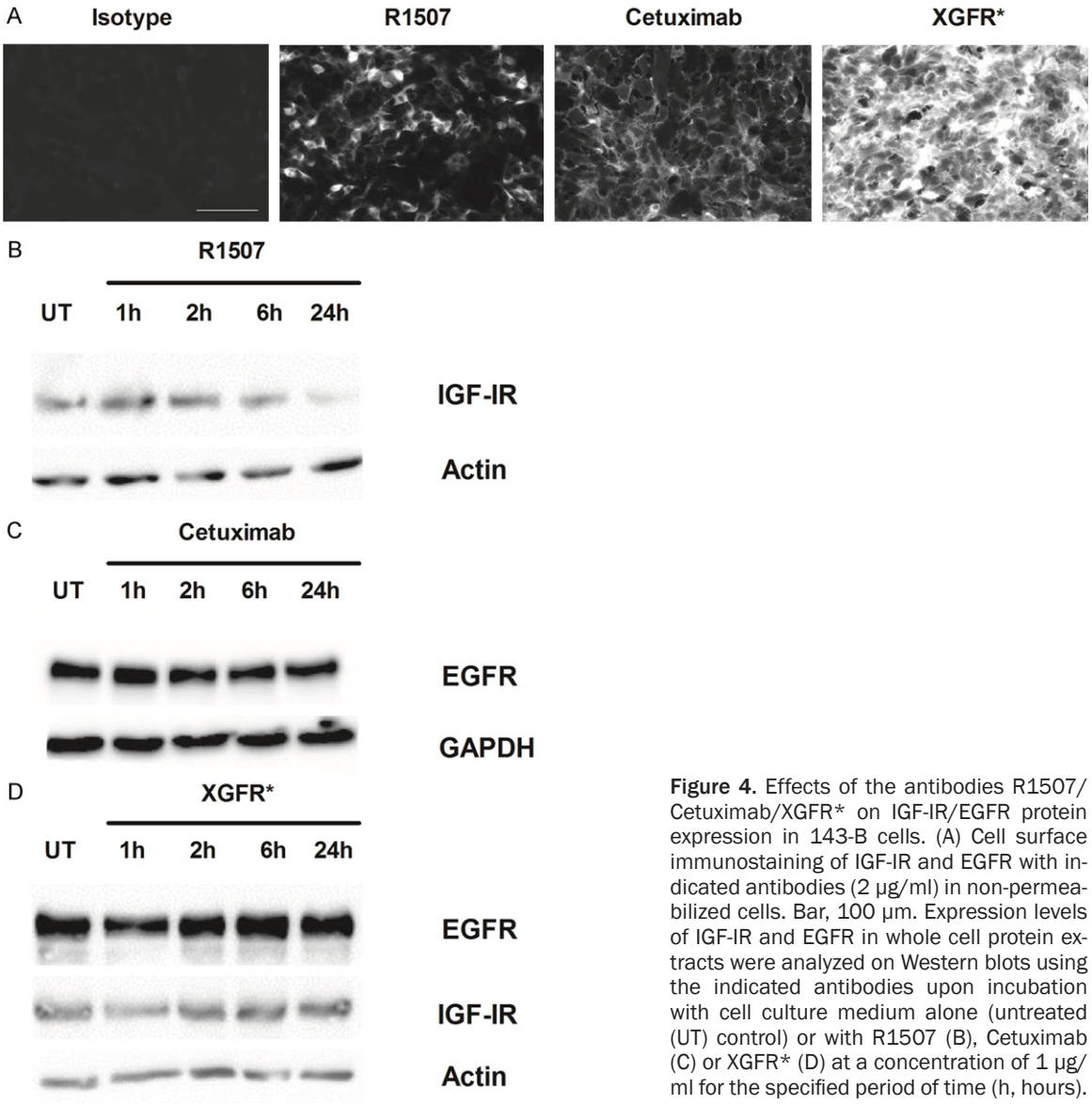

Figure 4. Effects of the antibodies R1507/Cetuximab/XGFR* on IGF-IR/EGFR protein expression in 143-B cells. (A) Cell surface immunostaining of IGF-IR and EGFR with indicated antibodies (2 μg/ml) in non-permeabilized cells. Bar, 100 μm. Expression levels of IGF-IR and EGFR in whole cell protein extracts were analyzed on Western blots using the indicated antibodies upon incubation with cell culture medium alone (untreated (UT) control) or with R1507 (B), Cetuximab (C) or XGFR* (D) at a concentration of 1 μg/ml for the specified period of time (h, hours).

Taken together, in 143-B cells all mono- and bispecific antibodies to IGF-IR and EGFR inhibited ligand stimulated signaling of respective receptors with a remarkable potency. In SaOS-2 cells, on the other hand, only R1507 and XGFR* were able to inhibit ligand-stimulated signaling and to a considerably lower extent than that observed in 143-B cells.

Effects of R1507, Cetuximab and XGFR on in vitro viability and migration of osteosarcoma cells*

In order to investigate the potential effects of IGF-IR and EGFR-targeting antibodies on cell viability we performed a WST-1 assay. The treatment of 143-B cells and of SaOS-2 cells with increasing concentrations of R1507, Cetuximab or XGFR* for 72 hours had no effect on their viability as shown in **Figure 3A** and **3B**, respectively.

We next explored if the antibodies affect tumor cell migration using a transwell migration assay. The migration rate of 143-B cells was significantly reduced compared to control in the presence of all antibodies as shown in **Figure 3C** ($P<0.001$). R1507, Cetuximab, a mixture of R1507 and Cetuximab or XGFR* decreased the migration rate to 56.2 ± 5%, 51.3 ± 3%, 34.2 ± 3.3% and 48.7 ± 7.1% when compared to that of untreated control, respectively. Treatment of

SaoS-2 cells with R1507, combination of R1507 and Cetuximab or with XGFR* revealed a less prominent effect on migration rates than in 143-B cells. Overall the migration rates were reduced to approximately 65% of that observed in untreated cells (**Figure 3D**, P<0.05). SaOS-2 cells treated with Cetuximab also showed a tendency to reduced migration (**Figure 3D**, P>0.05).

Collectively, these results demonstrate that targeting IGF-IR and EGFR had no impact on *in vitro* cell viability, but led to the reduction of the migratory potential of osteosarcoma cells.

Binding of R1507, Cetuximab and XGFR to 143-B cells and their effect on IGF-IR or EGFR protein levels*

Since, in the so far described *in vitro* experiments, the responses of 143-B cells to mono- and bispecific IGF-IR and EGFR blocking antibodies were more robust than those observed in SaOS-2 cells, we decided to use a 143-B xenograft model for our *in vivo* preclinical study and therefore further characterized *in vitro* the interaction and resulting effects of the antibodies in 143-B cells. Immunocytochemistry confirmed binding of the antibodies to the cell surface (**Figure 4A**). Interestingly, binding of XGFR* to cell surface IGF-IR and EGFR receptors appeared considerably more efficient than that of the monospecific antibodies.

Previous studies have shown that cell surface targeting of IGF-IR and EGFR by antibodies can lead to a decrease of receptor protein expression levels through receptor internalization and subsequent degradation. Therefore, we investigated IGF-IR and EGFR protein levels by Western blotting upon incubation of 143-B cells with R1507, Cetuximab or XGFR* in a time course experiment. R1507 provoked downregulation of IGF-IR in 143-B cells after 24 hours of incubation (**Figure 4B**). In contrast, protein levels of EGFR or IGF-IR remained unchanged upon Cetuximab- or XGFR* treatment (**Figure 4C** and **4D**).

In summary, the bispecific XGFR* antibody exhibited the most potent binding to 143-B cells. In contrast to Cetuximab and XGFR*, which did not affect protein levels of respective receptors, R1507 reduced IGF-IR protein expression levels.

XGFR inhibits intratibial primary tumor growth and suppresses pulmonary metastasis in a xenograft osteosarcoma mouse model*

The intratibial 143-B xenograft osteosarcoma mouse model that closely recapitulates human metastatic disease was used to evaluate and compare *in vivo* the response to IGF-IR and EGFR targeting therapy with respective monospecific or bispecific antibodies. Treatment of the mice by weekly intraperitoneal injections of the antibodies or vehicle (saline) was initiated on day 10 after intratibial tumor-cell injections (TCI) (**Figure 5A**). Three injections per mouse were given in total. Tumor-bearing mice in respective experimental groups received 10 mg per kg of body weight of R1507 or 10 mg/kg Cetuximab alone or 10 mg/kg of R1507 together with 10 mg/kg Cetuximab or 20 mg/kg XGFR* alone. XGFR* was applied at 20 mg/kg to ensure equimolar dosing between XGFR* and R1507/Cetuximab combination. Development over time of primary tumors associated with osteolytic lesions in the mice tibias was monitored by X-Ray imaging. The X-Ray images of injected legs of vehicle- or XGFR*-treated mice are presented in **Figure 5B**. Primary tumor growth, assessed by caliper measurements, was significantly inhibited by Cetuximab and XGFR* treatment, as demonstrated by a 20% and 40% decrease in the final primary tumor volume when compared to that of the vehicle-treated group, respectively (**Figure 5C**; P<0.05, P<0.001). Interestingly, R1507 alone or in combination with Cetuximab, did not have any effect on primary tumor growth.

In addition to the tumour suppressive effect, XGFR* had a significant anti-metastatic activity, demonstrated by quantification of *ex vivo* X-Gal stained metastases on lung surfaces (**Figure 6A** and **6B**). Animals treated with XGFR* displayed 2.2 fold lower mean numbers of pulmonary metastases than the vehicle-treated animals (**Figure 6B**, P<0.05). R1507, Cetuximab or R1507/Cetuximab combination, on the other hand, did not significantly alter the number of pulmonary metastases, although we observed a tendency towards a decreased number of metastases in Cetuximab and R1507/Cetuximab treated groups.

In summary, therapy with bispecific XGFR* had a more pronounced inhibitory effect on tumor

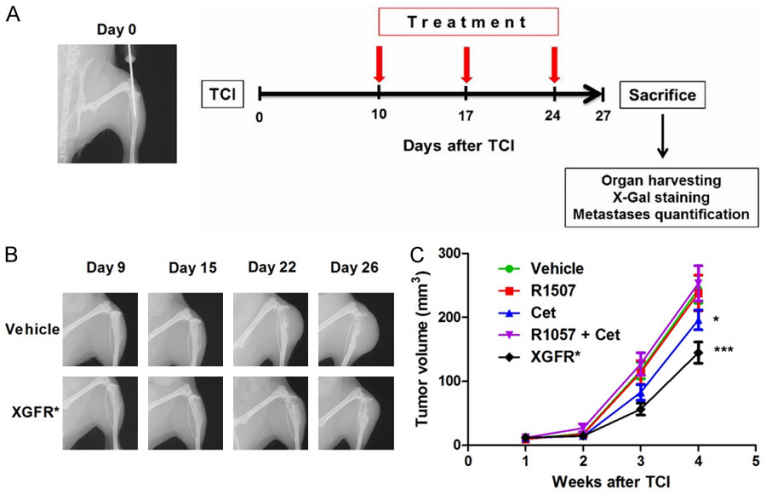

Figure 5. Effects of IGF-IR and EGFR targeting antibodies on primary tumor growth in the 143-B xenograft osteosarcoma mouse model. A. X-Ray image of the intratibial injection of 143-B cells into a SCID mouse taken at the time of the tumor cell injection (TCI) on Day 0 (left panel). Study design and treatment schedule of tumor bearing mice included weekly intraperitoneal injections of vehicle (control), R1507 (10 mg/kg body weight), Cetuximab (10 mg/kg), R1507 and Cetuximab (10 mg/kg + 10 mg/kg) or XGFR* (20 mg/kg) (right panel). B. Primary tumor development over time monitored by X-Ray imaging of tumor bearing legs at indicated time points in mice treated with vehicle or XGFR* antibody. C. Intratibial tumor growth over time examined by caliper measurements. Values are the mean ± SEM of the data collected from 12 vehicle-, Cetuximab-, R1507 + Cetuximab- and 10 R1507- and XGFR* treated mice; *, P<0.05; ***, P<0.001.

growth and pulmonary metastasis than the treatment with the monospecific IGF-IR or EGFR antibodies or a combination thereof.

XGFR treatment is associated with increased natural killer cell infiltration into primary tumor tissue*

Our *in vivo* therapy study revealed that the combination of targeting both IGF-IR and EGFR with a single bispecific antibody has evidently superior anti-tumor and anti-metastatic efficacy than the dosing of individual monospecific antibodies, although the blocking capacity of ligand-activated signaling *in vitro* was similar for all antibodies. We, therefore, hypothesized that antibody-dependent immune effector functions may contribute to XGFR* *in vivo* effectiveness. Hence, we investigated the presence of tumor-infiltrating natural killer (NK) cells by immunohistochemistry on primary tumor tissue sections using a pan-NK anti-mouse CD49b antibody (**Figure 7A**). We found a significantly increased accumulation of NK cells in primary tumors of XGFR*-treated mice than in all the other treatment groups (**Figure 7B**, P<0.05).

Taken together, the data presented here indicate an association between XGFR* treatment and infiltration of the innate immune system effector cells into the tumor microenvironment.

Discussion

Due to its high propensity for metastatic spread, osteosarcoma is the second leading cause of cancer-linked death in childhood and adolescence [39]. Therefore, novel therapeutics, efficiently preventing metastatic disease, are mandatory in order to improve the currently poor patients' survival.

Although initial studies using IGF-IR-targeting antibodies showed promising potential for the treatment of patients with bone and soft tissue sarcomas, phase II clinical trials revealed discouraging results [12-14]. Activation of alternative signaling pathways mediating resistance to single agent therapy might underlie the reported variable response rates. Along these lines it has been demonstrated in other cancer types that signaling through EGFR may overcome resistance to IGF-IR inhibitors and vice versa [40, 41], implying that co-targeting these two receptor tyrosine kinases might provide more clinical benefit to osteosarcoma patients.

In the present study we investigated *in vitro* and *in vivo* effects of IGF-IR and/or EGFR targeting by the antibodies R1507, Cetuximab and XGFR* in osteosarcoma. First, we were able to confirm the previously reported findings that osteosarcoma cells express functional IGF-IR and EGFR receptors [20, 42-45]. Ligand-induced downstream signaling was efficiently suppressed by monospecific antibodies and by XGFR*, most prominently in 143-B cells, as demonstrated by reduced Akt phosphorylation. These results are in line with the findings in lung and colon carcinoma as well as in Ewing sarcoma cell lines [28, 29]. Despite the effective inhibition of receptor activation and R1507-provoked IGF-IR downregulation, *in vitro* cell

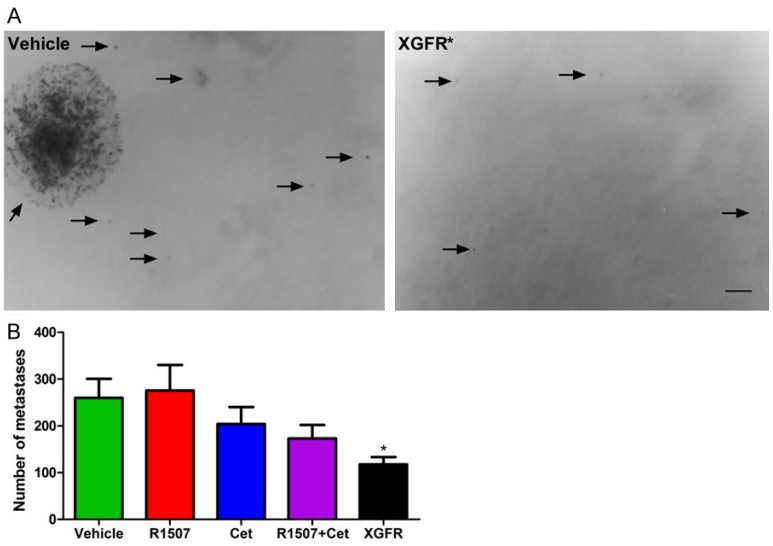

Figure 6. Effects of IGF-IR and EGFR targeting antibodies on lung metastasis in the 143-B xenograft osteosarcoma mouse model. A. Representative photos of lungs with X-Gal stained metastases (arrows) in indicated treatment groups. Bar, 250 μm. B. Quantification of pulmonary metastases on the lung surface of mice receiving vehicle (n=12), R1507 (n=10), Cetuximab (n=12), combination R1507 + Cetuximab (n=12) or XGFR* (n=10). The lungs were harvested and subjected to X-Gal staining 27 days after tumor cell injection. Values are the mean ± SEM; *, P<0.05.

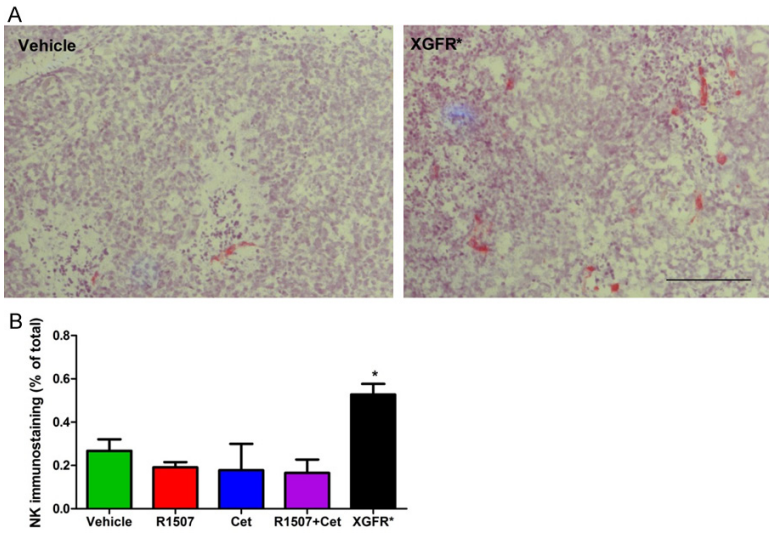

Figure 7. Infiltration of natural killer cells into 143-B xenografts. A. Detection of natural killer (NK) cells by immunohistochemistry (CD49b; red staining). Representative images of primary tumor sections obtained from vehicle- or XGFR*-treated mice. Cell nuclei counterstained with hematoxylin (purple staining). Bar, 200 μm. B. Quantification of NK immunostaining in primary tumor sections of mice receiving vehicle (n=3), R1507 (n=3), Cetuximab (n=3), combination R1507 + Cetuximab (n=3) or XGFR* (n=3) treatment. Values are the mean ± SEM; *, P<0.05.

viability was not affected, much like in a number of various cancer cell lines [46, 47]. However, our *in vivo* preclinical study revealed diverse responses to the mono- and bispecific antibodies. R1507 was ineffective in inhibiting growth and tumor cell dissemination, which is consistent with the restricted efficacy of this antibody seen in the clinical trials [14]. Different from these observations are the findings of a study in which R1507 was shown to delay tumor growth in four out of six osteosarcoma cell line xenograft models [11]. It is however noteworthy that the above mentioned animal studies employed subcutaneous osteosarcoma mouse models, as opposed to our *in vivo* study in which we made use of an intratibial osteosarcoma mouse model, supporting the idea that our orthotopic model might have more translational relevance. In contrast to R1507, Cetuximab displayed a modest anti-tumor effect *in vivo* concordant to the previous report investigating effects of Gefitinib, a selective EGFR tyrosine kinase inhibitor, and concluding that EGFR is not a major driver of osteosarcoma cell growth *in vitro* [43]. Interestingly, the combination of R1507 and Cetuximab did not affect tumor growth; therefore, we speculate that there might be the possibility of decreased binding of Cetuximab to tumor cells *in vivo* in the presence of R1507. Simultaneous IGF-IR and EGFR targeting with XGFR*, on the other hand, prominently inhibited primary tumor growth. Moreover, our preclinical study demonstrated for the first time that XGFR* exhibits an anti-metastatic activity. Because all antibodies decreased motility of osteosarcoma cells *in vitro*, but only

XGFR* significantly reduced the number of lung metastases, we believe that the *in vivo* metastatic inhibition caused by XGFR* is not a consequence of a direct effect of XGFR* on tumor cell capacity to migrate. We provide evidence that XGFR* most potently binds to 143-B cells *in vitro* compared to the monospecific antibodies. Furthermore, XGFR* does not induce receptor internalization and degradation of the respective receptors in osteosarcoma cells. The prolonged display of XGFR* on cell surface may lead to increased ADCC as seen in other cancer cell models [28]. Along these lines, we showed that NK cells accumulate in the intratumoral microenvironment of XGFR*-treated animals. We, therefore, postulate that the therapeutic response to XGFR*, reflected in the inhibition of primary tumors and metastatic spread, is associated with the recruitment of immune effector cells and subsequent immune system-mediated cell killing.

Our data once again underline the remarkable advantage of bispecific antibodies that, in addition to interfering with two different targets, serve as tumor-targeted immune cell recruiters [26]. The bispecific antibodies have a great potential in the oncology field and their combination with other treatment modalities including chemotherapy should be explored.

In conclusion, our study highlights the bispecific anti-IGF-IR/EGFR antibody XGFR* as a novel effective therapeutic option against metastatic osteosarcoma and provides the rationale for future investigations in clinical settings.

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Disclosure of conflict of interest

None.

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